Long-lived antitumor $CD8^+$ T cells in Melanoma patients who had complete response to checkpoint blockade immunotherapy

by

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A thesis submitted to Johns Hopkins University in conformity with the requirements for the degree of Master of Science.

Baltimore, Maryland

April 2018

Abstract

The emerging capabilities of checkpoint blockade immunotherapy to potentiate effective antitumor immune responses has galvanized the field of cancer treatment. Initial usage of checkpoint blockade immunotherapy has already shown great promise, driving increases in overall survival and progression free survival (PFS) in patients with non-small cell lung cancer and melanoma[1-2]. However, they are incompletely ineffective in different patients and cancer types[3-5]. Studies have shown that checkpoint blockade immunotherapy in combination with strategies to induce T cell targeting of tumor-specific antigens and mutation-associated neoantigens, demonstrates the ability to illicit stronger tumor regression[6]. Using an assay optimized by the Pardoll lab, we looked to identify TAA-/MANA-specific $CD8^+$ TCRs in patients who successfully had complete tumor regression in response to checkpoint blockade immunotherapy, anticipating that there may be underlying patterns or preferences in T cell targeting of tumor antigens. Modeling for long-lived $CD8^+$ T cell memory, we ran the FEST assay using TAAs and MANAs on $CD8^+$ T cells isolated from 3-year post-treatment peripheral blood from metastatic melanoma patients who had complete responses to checkpoint blockade immunotherapy. We identified 7 antigen-specific $CD8^+$ TCR clonotypes which contain specificities for TAA or putative MANA peptides. Interestingly, we observe multiple T cell clones with different underlying nucleotide sequences contributing to the same amino acid TCR sequence with typically one nucleotide TCR clone dominating contribution to the clonotype. Identified TCR clonotypes have specificities for antigens of recurrent mutations or mutations in proteins that carry prognostic value. We also have discovered the presence of our identified MANA-specific TCR clones in tumor infiltrating lymphocytes of melanoma patients from

separate cohorts. Our data suggests the potential enrichment for TCRs which selectively recognize mutations in proteins that provide selective advantage or in proteins carrying passenger mutations. With the presence of both, functionally responsive TAA-specific and MANA-specific T-cells, in the long-lived $CD8^+$ TCR repertoire of metastatic melanoma patients who had complete responses to immunotherapy treatment, our study demonstrates the importance of targeting both types of antigens in mounting an effective antitumor immune response.

Primary Reader: Drew Pardoll M.D./Ph.D.

Secondary Reader: Jay Bream Ph.D.

Acknowledgments

I first need to thank my mentors Dr. Drew Pardoll and Dr. Jay Bream for guiding me on my project and giving me invaluable advice as I continue my journey. Next, a big shout out to Kellie Smith, Haidan Guo, Justina Caushi, Vanessa Chan, Prerna Suri, Arbor Dykema, Jiajia Zhang, John-Williams Sidhom, Margueritta El Asmar, Lin Lin for helping me along my way.

To my friends and colleagues, I've gotten to know the past few years as we struggled and supported each other, I wish you guys well! Guarav Dhiman, Emily Hendrickson, Madhura Kulkarni, Monali Praharaj, Emily Thompson, Raghav Vij, Ross Warner.

Finally, I have to thank my family, for whom I get my strength and confidence to keep pursuing my dreams. Mourning the losses of multiple loved ones this past year, our love and support for each other never wavered and there are no words to describe how thankful I am to have you.

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A. Introduction

Melanoma is the most aggressive form of skin cancer, although contributing to only 1% of total cancer cases, its worldwide incidence has been steadily increasing for the past 3 decades[9,10]. Sun exposure and having fair skin are regarded as risk factors for developing melanoma, presumably due to mutations brought on by ultraviolet radiation induced DNA damage[10,11]. If caught and treated during early stages, melanoma patients have a 99% 5-year survival rate, in contrast to a 10-15% 5-year survival rate when caught at later stages[12,13]. However, with the emergence of checkpoint blockade immunotherapy as an effective cancer treatment for patients with metastatic melanoma, has resulted in dramatic increases in objective response rates (ORR), with 40-50% of metastatic melanoma patients seeing measurable tumor regression after receiving immunotherapy. In addition to increased ORR, duration of progression free survival of these patients has increased to about 12 months[1]. Despite the observed efficacy of checkpoint blockade immunotherapy, only a subset of patients respond favorably to treatment and achieve sustained tumor remission[14]. As the field of cancer immunotherapy continues its rapid expansion, it becomes imperative to characterize the nature of antitumor immune responses to address discrepancies in checkpoint blockade immunotherapy treatment responses[15].

An individual's immune system is a highly capable defense system which enables the recognition and destruction of infected or otherwise foreign and transformed self cell. Generally, immunity consists of innate immune responses against nonspecific pathogen targets, and adaptive immune responses against targets with high specificity. Both arms working in concert in contributing to overall immune responses to foreign targets, adaptive components

such as T and B lymphocytes are primarily responsible for mediating activated immune responses against cellular targets with high degrees of specificity. Each T cell specificity is derived from the surface expression of unique T cell receptors (TCRs). Each TCR is a heterodimer of randomly expressed TCR-α and TCR-β chains, with variable regions from each chain contributing to antigen-binding specificity. Stochastic genetic recombination events drive expression and diversity of associated variable/diversity/joining genes for each TCR chain which then heterodimerize. This process can generate a TCR sequence from a repertoire of 10^{15} to 15^{20} possible unique TCRs, allowing T cells to potentially recognize an immense spectrum of antigens[17]. After successful TCR formation, T cells further mature to express either $CD4^+$ or $CD8^+$ glycoproteins on their surfaces. Generally, $CD4^+$ "helper" T cells aid in further immune activation while $CD8^+$ cytotoxic T lymphocytes (CTLs) cellular killing abilities. $CD4^+$ and $CD8^+$ T cells disseminate throughout the body, continuing surveillance of antigenic peptides displayed on cell surface major histocompatibility complex (MHC) class I and class II molecules, respectively. After successful activation upon recognition of their TCR's cognate peptide, $CD4^+$ and $CD8^+$ T cells can further differentiate into active effector or memory T cells[16]. With antigen availability being a determining factor, prolonged antigen exposure associated with development of this small subset of long-lived T cell memory. These memory T cells survive and circulate for extended periods of time, poised to react quickly upon repeated exposure to its cognate antigen[16,30].

T cells are key activators, regulators, and effectors of adaptive immune responses, however, their functionality hinge on their ability to activate upon recognition of cognate antigen-MHC complexes on cell surfaces. In addition to initial TCR and antigenic peptide-MHC binding, T cells need proper interaction of costimulatory receptors as well as activating

cytokines. Overall co- stimulation is a culmination of co-stimulatory and co-inhibitory receptor signals received when a T cell interacts with a target cells antigen-MHC complex. Depending on T cell lineage, various effector responses can occur. In terms of activated CTL effectors, they directly destroy cells display antigens for which their TCRs carry specificity. To prevent harmful T cell reactivity if expressed TCRs carry specificities for host, self-antigens, central and peripheral tolerance mechanisms play a fundamental role. A common immunotolerance mechanism is the expression of costimulatory molecules (co-inhibitory) which directly inhibit T cell activation. However, although this may be a normal process, tumors also use this mechanism as a means of suppressing activation of immunity against tumor antigens[18,19].

T cell immune-checkpoint

T cell immune-checkpoint molecules are subsets of coinhibitory receptors/ligand complexes fundamental in downregulating T lymphocyte activation to induce tolerance. While fundamental in suppressing self-reactive lymphocytes under normal circumstances, immune-checkpoints can be overexpressed in tumors due to dysregulation brought on by malignant transformation, providing protection from antitumor T cell-mediated immunity. Subsequently, cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death 1 (PD-1) are amongst the most extensively studied T cell immune-checkpoint receptors and are the targets of current approved cancer immunotherapies[18-20].

Mainly affecting the priming step of naïve T cell activation occurring in lymphoid tissues, T cell expression of the inhibitory receptor CTLA-4, outcompetes its own costimulatory receptor CD28 with stronger binding of CD80/86 ligands expressed on antigen presenting cells. When CTLA-4 receptor binds its ligand, it triggers a cascade of inhibitory events within the T cell,

suppressing proliferation and inducing (anergy). While CTLA-4 mainly inhibits T cell priming steps in lymphoid tissues, the PD-1 receptor generally inhibits activation of mature T cells upon binding programmed cell death ligand 1 (PD-L1) expressed on target cell surfaces. CTLA-4 is considered constitutively expressed on T cells, however, PD-1 expression is induced upon T cell activation and it can also be expressed on lymphocytes other than T cells. T cell expression of PD-1 receptors is a hallmark of an "exhausted" state, characterizing T cell dysfunction. CTLA-4 has been implicated in the induction of immunosuppressive tumor microenvironments. Both immune-checkpoint receptors work to inhibit T cell activation, inducing immunotolerance to tumor antigens and suppressing antitumor immune responses[18-20].

After initial discoveries that tumor take advantage of these checkpoints, immunotherapies were quickly developed using monoclonal antibodies (mAbs) which specifically target and block PD-1 and CTLA-4 checkpoint activation. Current approved checkpoint blockade immunotherapy treatments for metastatic melanoma patients include a nivolumab (anti-PD1 mAb) monotherapy or nivolumab/ipilimumab (anti-PD1/anti-CTLA4 mAbs) combinatorial therapy[20]. These treatments quickly achieved FDA approval, after displayed efficacy with about 50% of melanoma patients receiving measurable tumor regression in initial checkmate clinical trials[14,20,21]. However, despite initially taking the cancer field by storm, complications began to surface as checkpoint blockade immunotherapy was inconsistently effective across different cancer types and in some cases, treatment resistance developed[5]. As various biomarkers and characteristics are investigated for prognostic value in predicting tumor response to immunotherapy, focus has shifted back to the bread and butter of the immune

system, back to what makes it so special: the ability to discriminate between self and foreign antigens.

Tumor-specific antigens vs. Tumor-associated antigens

Possessing the potential to generate antigen-specificities from over a billion possible TCR sequences, endogenous patient T cell repertoires should conceivably be able to target expressed tumor antigens. As tumors continue accrue mutations to their genomic DNA, a hallmark of all cancers, their proteomic expression profiles inevitably change[22]. When displayed on cell surface MHC molecules, peptides derived from altered proteomes can potentially be recognized as immunogenic by endogenous T cells, eliciting an immune response.

Known to express different antigen profiles than normal counterparts, expressed tumor antigens generally come in two flavors, tumor specific antigens (TSAs) and tumor associated antigens (TAAs). TSAs, also called neoantigens, are mutated forms of self-antigens, absent from normal antigen repertoire and specifically expressed in tumors[23]. As many of these neoantigens are due to nonsynonymous mutations in original genomic code of tumor cells, the term mutation-associated neoantigens (MANAs) is also often used, unless occurring in driver genes, MANAs are generally considered patient-specific[24,25]. In contrast, TAAs are self-antigens expressed by the tumor, but also expressed in normal cells to a limited degree. Examples of TAAs can be from germline/testis and differentiation proteins such as *MAGE* and *MART-1*, respectively, found overexpressed in melanoma[26]. Since melanoma antigen *MART-1* and testis antigen *MAGE* were some of the first antigens found to be selectively expressed in melanoma, they were among the first targets of T cell therapy[26]. Also, with the observation that increased mutational load and/or neoantigen load is significantly associated with response to checkpoint

blockade immunotherapy, researchers have worked to identify neoantigen targets as well[24,25]. Studies using T cell targeting therapies against MANAs or TAAs have shown promise, however, cite the potential problem of treatment resistance, implying that immune antitumor mechanisms need further elucidation[24-26].

Antigen-specific T cells

Due the breadth of their TCR repertoires and direct cytolytic function, CTLs expressing tumor antigen-specific TCRs have the ability to carry out direct killing of tumor cells[27]. Although evidence implies that this does indeed occur, tumors can avoid $CD8^+$ T cell-mediated immune destruction by adopting a variety of immune evasion mechanisms, these of which can generally be divided into two groups: 1) the induction of an unresponsive, immunotolerant state and 2) evasion of activated immune effector cells[19]. As mentioned previously, current checkpoint blockade immunotherapies help overcome tumor-induced immunotolerance, unleashing T cell-mediated immune responses. The next obstacle to producing effective antitumor immunity involves the second aforementioned cancer immune evasion strategy, which presents the problem of effectively targeting potentiated T cell immunity against the tumor. With this is in mind, considerable effort has been invested in mapping intratumor mutation and neoantigen landscapes[28,29]. While antigen identification is undoubtedly important, it's imperative to also identify the nature of activated T cell responses[31]. T cell activation effectively targets cancer destruction working under the assumption that endogenous TCR repertoires contain specificities for tumor antigens. Characterizing patterns or preferences in antigen targeting of endogenous TCRs, may provide invaluable information on what types of antigens illicit the most advantageous antitumor immune response[24-27,29].

Recent advances in next generation sequencing (NGS) and analytical techniques has allowed more accurate interrogation of expressed tumor antigens in addition to the T cell repertoire which recognizes them[8]. With these techniques, our lab has established the FEST assay which accurately and faithfully identify low-frequency TCR clonotypes which functionally expand to specific antigen stimulation[8]. This assay not only detects antigen-specific clonotypes at higher sensitivity and specificity than traditional immunoassays, but also produces a readout of unique TCR sequences which functionally respond[31,32]. Using amino acid and nucleotide TCRVβ CDR3 sequences to adequately barcode unique $CD8^+$ TCR clones, abundance of each unique TCR are tracked in response to different peptide stimulation[32]. Since T cells fundamentally proliferate upon antigen-dependent activation, this assay identifies antigen-specific TCRs based off clonal population expansion of TCR clonotypes in response to peptide stimulation.

Previous experiments used this assay design to identify MANA-specific TCR clones in NSLCLC patients who received anti-PD1 immunotherapy. Pairing FEST analysis with longitudinal whole exome sequencing data of NSCLC tumors, they were able to map tumoral loss of neoantigens that were specifically targeted by endogenous TCRs. They observed that neoantigen and mutational landscapes alone could not predict response to checkpoint blockade immunotherapy, implying that antigen quality have more influence over antigen quantity[8].

The wealth of information produced while performing this assay, presents the unique opportunity to interrogate TCR-recognized tumor antigens as well as the functionally responding T cell repertoire. Not only can this information be used to evaluate TAA versus MANA $CD8^+$ TCR targeting, but also evaluate motifs enriched in identified antigen-specific TCRs. Differentiation

into long-lived $CD8^+$ memory T cell is characteristic of $CD8^+$ T cells functionally responding recognition of its TCR's cognate antigen[16,30]. Evaluating the tumor antigen-specific T cell repertoires isolated from peripheral blood samples collected long after their last immunotherapy treatment, enables us to interrogate long-lived $CD8^+$ memory T cells. Interrogating long-lived antigen-specific TCR repertoires from patients who had complete responses to immunotherapy, enables us to potentially further characterize these reactive TCRs as contributors to effective antitumor immunity.

B. Project Aims

I. Identify putative mutation-associated neoantigens (MANAs) expressed in tumors of metastatic melanoma patients.

II. Identify TCRVβ CDR3 sequence clones which functionally expand to specific MANA- or TAA-peptide stimulation.

III. Further characterize antigen-specific TCR clonotypes based on amino acid and/or nucleotide TCR sequences.

- Assess potential differences in characteristics between MANA/TAA specific TCR clonotypes.

IV. Identify intratumoral expression of immunological markers to phenotypically assess the tumor microenvironment.

C. Methods

Patient Samples

Melanoma patients with pathologic complete tumor responses to checkpoint blockade immunotherapy were selected from a cohort of metastatic melanoma patients enrolled in J13139 tissue collection protocol at Johns Hopkins Hospital who received standard of care treatments with PD-1 (nivolumab) monotherapy or PD-1 and CTLA-4 (Nivolumab and ipilimumab) combinatorial therapy. Selection was also based on availability of initial pre-therapy tumor specimens and frozen PBMCs from a shared timepoint post therapy. In compliance with HIPAA regulations and protocol guidelines, all patient information (name, age, sex, race, comorbidities, etc.) was de-identified or not given to us.

Per clinical study protocol guidelines, patient tumors were resected pre-treatment (when applicable), while blood was drawn at various timepoints during and after checkpoint blockade immunotherapy. For uniformity, patients with both pre-treatment tissue samples and a 3-year post-treatment blood draw were included. Study investigators collected whole blood purple top EDTA tubes and processed the same day for PBMC isolation. PBMCs were isolated using Ficoll-Paque (Sigma-Aldrich) protocols solution and stored in 10% DMSO at -80°C.

MANA and TAA selection

Whole exome sequencing (WES) of matched patient tumor/normal specimens was performed using PGDX ImmunoSELECT™-R platform (Baltimore, MD). ImmunoSELECT™-R analysis and bioinformatics pipeline identified somatic nonsynonymous mutations (SNPs/insertions/deletions), from which candidate putative neoantigen peptides (8-11 amino acid length) were selected.

WES data also included genotyping of HLA-alleles, allowing for accurate prediction of MHCI binding of putative neoantigen peptides. Neoantigen peptides were excluded if classified as non-binders to patient MHC I molecules (netMHCpan) or if IC50 <50nM. Top 10 candidates for each representative HLA-allele then selected based on ranking of associated somatic mutation frequency in SKCM tumors from TCGA database. Selected peptides are our putative MANA peptides.

We selected validated TAA peptides of known melanoma TAA proteins (gp100, MAGE-A3, Mart-1, Tyrosinase, PMEL) from online immune epitope database and analysis resource (iedb.org). Validated TAA peptides was based on number of positive results ($n \geq 5$) from T cell and/or MHC binding assays. Candidate TAA peptides were ranked based on how many previous studies they were tested in ($n \geq 5$). Top 10 candidate TAA peptides for each patient HLA-allele restriction were selected for synthesis. All peptides were synthesized using Sigma-Aldrich PEPscreen® platform (St. Louis, MO) as lyophilized peptides, which were dissolved in minimal DMSO and resuspended as 100 μg/mL aliquots in AIM V media, and stored at -80°C.

10-day T cell culture

Each plate only contained T cells from one patient. T cells were isolated from frozen patient PBMC samples using EasySep Human T Cell Enrichment Kit and EasySep purple magnet (Stemcell Technologies; Vancouver, Canada). T cell and a non-T cell lymphocyte fractions were washed and resuspended at a concentration of 2.0 $x10^6$ cells/mL in AIM V media (50 μg/ml gentamicin). Non-T cell fractions were irradiated at 3,000 γ-rads and washed to prevent proliferation and overcrowding. On a 96 well tissue culture-treated plate, irradiated non-T cell

and T cell fractions were added at a 1:1 ratio to a final volume of 250 uL per well. Before plating, an addition well's equivalent of T cells is frozen down as baseline pre-culture reading. Representative MANA, TAA, CEF pool, SL9 peptides were individually added to separate wells at a final well concentration of 1ug/mL, leaving one well peptide-free. CEF pool was used as a positive control, while SL9 of irrelevant HLA-type was used as negative priming control. On day 3 half of well culture media is replaced with fresh AIM V serum free medium (50 μg/ml gentamicin) (ThermoFisher) along with addition of cytokines IL-2 (Chiron, IL-7 (Miltenyi), and IL-15 (Peprotech) for final well concentrations of 50 IU/mL, 25ng/mL, and 25 ng/mL respectively. On day 7, half media was replaced again with fresh AIM V (50 μg/ml gentamicin) along with IL-2, IL-7, IL-15 for final well concentrations of 100 IU/mL IL-2, 25ng/mL IL-7, 25 ng/mL IL-15. On day 10, $CD8^+$ T cells were isolated from T cell cultures using EasySep Human $CD8^+$ T cell Enrichment Kit EasyPlate EasySep Magnet (Stemcell Technologies).

DNA extraction was performed on isolated $CD8^+$ T cells using QIAamp DNA micro kit and protocol (Qiagen). Extracted $CD8^+$ T cell DNA frozen at -80°C and sent to Adaptive Biotechnologies immunoSEQ® (Seattle, Washington) for TCRVβ CDR3 sequencing.

Tumor infiltrating lymphocyte extraction

Resected pre-treatment formalin-fixed paraffin-embedded (FFPE) tumor sections were fixed onto positively charged slides (5-10 slides). Pathologist identified tumor infiltrating lymphocytes from hematoxylin and eosin (H&E) stained slides. Using H&E stained slides as a template, infiltrating lymphocytes were scraped from FFPE unstained positively charged slides into 1.8mL conicals, from which DNA extraction was performed using High Pure FFPET DNA

Isolation Kit (Roche). Extracted DNA stored at -80°C prior shipping to Adaptive Biotechnologies immunoSEQ® (Seattle, Washington).

Biostatistical Analysis of significant antigen-specific expansions

With T cells from only one patient tested per plate, analysis of antigen-specific expansions is done per plate. TCRVβ CDR3 amino acid and nucleotide sequencing data was obtained through Adaptive Biotechnologies Immunoseq® platform and used to barcode unique TCRs clonotypes. Fisher's exact test was used to compare relative enrichment of productive TCR templates between a peptide condition and control well (no peptide) condition that received – peptide/ + cytokine stimulation. With Benjamin-Hochberg procedure, p-values were adjusted to account for baseline threshold (n=1 TCR sequence template (nucleotide or amino acid sequence)), odds ratio threshold (OR=5), and normalized expansion compared to those of every other well condition (FDR threshold < .05). TCR clonotypes which satisfy these conditions are considered to have significant antigen-specific expansions, classifying them as antigen-specific TCRs.

D. Results

Mutagenic load, neoantigen load

Patient whole exome sequencing data provided information on mutational and neoantigen landscapes of patient's tumors before receiving checkpoint blockade immunotherapy. Distinct variation in mutational and neoantigen load was observed in the three melanoma patients tested. Patient 1 possessed the most natural germline variation (32,392 germline SNPs) while also expressing considerably more somatic mutation (1,422 somatic sequence alterations) than the other two patients (292 and 218 somatic sequence alterations) (**Table 1).** Little to no variation in gene copies were seen in patients 1 and 2 (0 and 2, respectively), while 49 copy number variations were seen in patient 3. Interestingly, patient 3 also had the lowest amount of somatic mutation (218 sequence alterations) of the three.

Patient	Mutation Based Tumor Purity	# of Somatic Sequence Alterations	# of Somatic Copy Number Alterations	Germline SNPs
Patient 1	49%	1,422	0	32,392
Patient 2	66%	292	2	30,408
Patient 3	41%	218	49	28,213

Table 1. Mutational and Neoantigen Tumor Landscape: # of somatic sequence alterations represent nonsynonymous genetic mutations in single genes resulting our candidate neoantigens from which we selected putative MANA-peptides (8-11 amino acid length).

As detailed in methods, Immunoselect-R platform accurately identifies somatic mutations and evaluates expression of neoantigen peptides based on predicted binding to patient HLA-allele restrictions. Our selected putative MANA-peptides, along with HLA-matched

TAA peptides were used to culture autologous T cells. Due to variation in T cell abundances between patient PBMC samples, number of MANA/TAA peptides conditions tested in each patient T cell culture differed (**Supplement Tables 1-4).**

Antigen-specific TCR clones

NGS data of TCRVβ CDR3 region genes was used to barcode unique TCR sequences at the amino acid and nucleotide levels. Using amino acid sequences, we tracked the template abundance of each unique TCR clonotype across different peptide well conditions after 10-day culture. We accurately identified significantly expanded TCR clonotype to specific peptide conditions using our optimized biostatistics analytic platform as stated in methods.

Significant antigen-specific expansions of CD8+ TCR clonotypes were observed in T cell cultures from one (patient 3) of the three patients (possible reasons for this in discussion). The T cell culture for patient 3 stimulated with 62 different MANA/TAA-peptides, from these, 6 MANA and 3 TAA peptides produced functional expansions of 11 unique antigen-specific TCR amino acid sequence clonotypes **(Table 2)**.

TCRVβ CDR3 Sequence (Amino Acid)	Protein (HLA-allele restriction	Tumor Antigen Type	Point Mutation	% Mutation Frequency	Sum frequency (%)	Fold Change (from Control)
CASSVGKGLG TEAFF	HUWE1 (HLA-A*02:01)	MANA	S115F	24%	0.421481	9
CASSPIAWDR DNSPLHF	HUWE1 (HLA-A*02:01)	MANA	S115F	24%	0.3706126	10
CASSHSGALN TEAFF	SEMA5A (HLA-A*33:05)	MANA	P483L	14%	0.3958510	33
CASSLALSETN NYGYTF	ZHX3 (HLA-B*51:01)	MANA	V447M	16%	0.3902510	5
CASSYLTGELF F	DLGAP1 (HLA-C*04:01 HLA-C*08:02)	MANA	E711G	16%	0.2289785	19
CASSFRMPEA FF	CPAMD8 (HLA-C*04:01)	MANA	E1249K	14%	0.1629229	14
CASSLLGGNQ PQHF	TTN (HLA-C*08:02)	MANA	E4247K	32%	0.5715082	48
CSAREGTANT EAFF	MART-1 (HLA-A*2:01)	TAA	N/A	N/A	0.3085651	14
CASSTSHLGW GYTF	MART-1 (HLA-A*2:01)	TAA	N/A	N/A	0.3085651	12
CASSPGQGSS GNTIYF	PMEL (HLA-A*2:01)	TAA	N/A	N/A	0.1437442	14
CASSKQGEGE KLFF	PMEL (HLA-A*2:01)	TAA	N/A	N/A	0.1698855 5	12

Table 2. Antigen-specific TCR clonotypes from patient 3: 7 MANA-specific and 4 TAA-specific TCR clonotypes were produced after T culture with associated tumor peptides. Point mutation denotes single amino acid substitution resulting from nonsynonymous nucleotide mutation. Mutation frequency is percentage of mutated reads at that given position. Sum frequency shows the percentage of total TCR reads from the associated peptide culture well that is due to the given antigen-specific TCR clonotype. Fold change quantifies the antigen-specific expansion of the given TCR clonotype compared to its abundance in the control which received cytokine stimulation without any peptide.

MANA-specific TCR clones

6 of the 52 tested MANA-peptides resulted in significant antigen-specific expansions of 7 TCR amino acid sequence clonotypes (**Supplementary Figures 1-6).** Antigen-specific TCRs recognized MANA-peptides from 5 of 6 patient HLA-allele restrictions. Many of the TCR-recognized MANAs resultant from nonsynonymous mutations in proteins (*HUWE1, SEMA5A, ZHX3, DLGAP1, TTN)* which have been identified to have various clinical and pathological relevance in melanoma patients and are addressed later in discussion.

Interestingly, 2 separate TCR clonotypes significantly expanded in the same *HUWE1* MANA-peptide culture (115S>F, HLA-A*2:01), while Single populations of TCR clonotypes expanded in other MANA-peptide cultures. The *HUWE1* MANA-specific TCR clonotypes combined for the highest frequency of all identified antigen-specific TCRs (measured individually in their associated peptide cultures), making up .79% of the total productive TCR templates in its culture well. However, when analyzed individually, *TTN* MANA-specific TCR was observed at the highest frequency (.572%). When cultured with its cognate *TTN* MANA-peptide (4247E>K, HLA-C*8:02), it produced the largest clonal antigen-specific TCR expansion in terms of raw abundance and amplification (82 templates and 42-fold change from control). Interestingly, of the MANAs which produced antigen-specific TCR clones, highest mutation frequency was also observed at the loci producing the *TTN* MANA. The *TTN* MANA-specific TCR clone was also calculated to have the most statistically significant antigen-specific expansion ($p>.10^{-16}$), followed by *SEMA5A* MANA-specific TCR clone ($p>.10^{-7}$). Multiple TCR clonotypes specifically recognizing the same *HUWE1* mutation, and strong clonal expansion of the *TTN* MANA-specific

TCR clonotype, suggests possible preference or importance in T cell targeting of these proteins or recognized peptide motifs.

TAA-specific TCRs

Since validated TAA peptides were lacking from other HLA-alleles, only HLA-A*2:01 restricted TAA peptides were tested in patient 3 T cell cultures. 3 of the 10 TAA peptide cultures resulted in expansion of 4 unique TCR amino acid sequence clonotypes **(Supplementary Figures 7-9).** Again, we observed 2 different TCR clonotypes significantly ($p > .001$) expand in the same *MART-1* peptide culture, while single TCR clonotypes expanded to 2 separate PMEL peptides (**Table 2**). As seen previously, two separate TCR clonotypes carrying specificity for the same peptide (MART-1) may demonstrate importance of targeting these antigens or recognized peptide motifs.

Nucleotide TCR sequences

Since multiple nucleotide (nt) sequences can encode for the same amino acid TCR clonotype, we identified true expansions of single TCR clone populations using their underlying TCRVβ CDR3 nt sequences. We found for the majority of antigen-specific TCR sequence clonotypes, multiple clonal populations of different nt sequences encoded for the same amino acid TCR sequence. Despite multiple TCR nt sequence clones encoding for the same amino acid sequence, one nt TCR clone seemed to dominate expansions of associated antigen-specific TCR clonotypes. Repeat FEST analysis using nt TCR sequences confirmed antigen-specific expansions in 10 of the 11 previously identified TCR clonotypes stemmed from a single TCR clone **(Table 3, Supplementary Table 5)**.

Peptide Type	Gene	Amino Acid TCRVβ CDR3	# Unique nt TCR clones
MANA	HUWE1	CASSVGKGLGTEAFF	2
MANA	HUWE1	CASSPIAWDRDNSPLHF	1
MANA	SEMA5A	CASSHSGALNTEAFF	2
MANA	ZHX3	CASSLALSETNNYGYTF	3
MANA	DLGAP1	CASSYLTGELFF	3
MANA	CPAMD8	CASSFRMPEAFF	1
MANA	TTN	CASSLLGGNQPQHF	5
TAA	MART-1	CSAREGTANTEAFF	1
TAA	MART-1	CASSTSHLGWGYTF	1
TAA	PMEL	CASSPGQGSSGNTIYF*	3
TAA	PMEL	CASSKQGEGEKLFF	1

Table 3. **Nucleotide TCR sequence clones:** Contribution of different nucleotide TCR clones to the same amino acid TCR. *= Expansion of single T cell clone not confirmed by nt TCR analysis

MANA-specific versus TAA-specific TCR clones

Average fold change of MANA-specific TCR clones (FC = 19.71) was higher than that of TAA-specific TCR clones (FC = 13), suggesting slightly stronger strength of response. More contributing nucleotide TCR clones tended to contribute to the same MANA-specific amino acid TCR clonotype as compared to number of contributing TAA-specific clones, with an average of 2.4 clones contributing to each MANA-specific TCR clonotype while an average of 1.5 clones contributed to TAA-specific TCRs. MANA-specific TCRs used TCRVβ27 the most, while TAA-specific TCRs were widely distributed **(Figure 1)**. Future directions of this study will further evaluate differences between MANA-specific and TAA-specific TCRs. We will determine motifs recognized by these antigen-specific TCRs to identify potentially enriched recognition motifs.

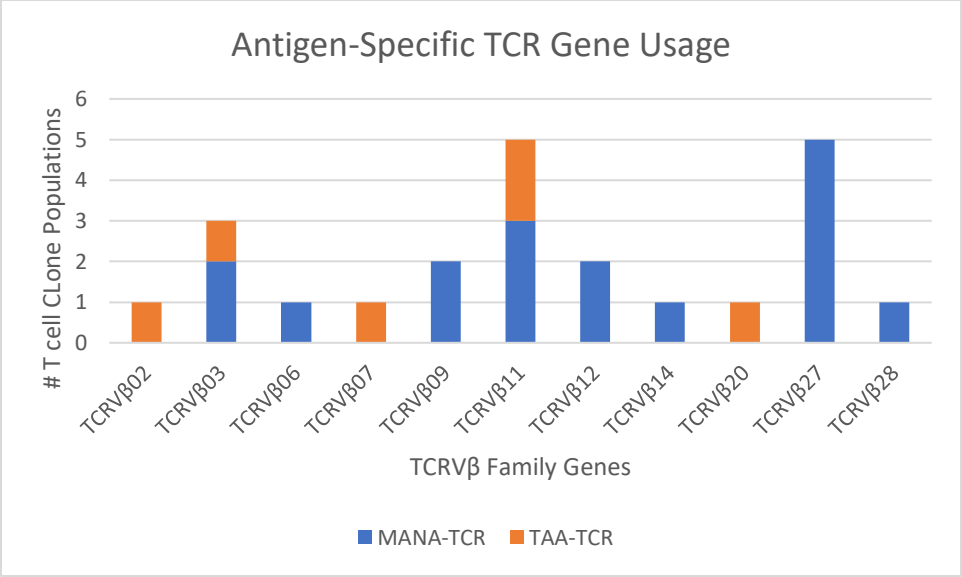

Figure 1: TCRVβ gene usage in antigen-specific TCRs. Using underlying nucleotide TCR sequences, the different clonal populations generated from each TCRVβ gene was summed.

Tumor-infiltrating T cells

NGS data of patient 3 tumor infiltrating T cells from showed highest usage of the TCRVβ 20 gene, followed by TCRVβ 06, TCRVβ 07, and TCRVβ 05 genes respectively (**Supplementary Figure 10)**. Unfortunately, we could not locate peripherally identified antigen-specific TCRs, in autologous tumor specimens, though this could be due to several reasons as mentioned later in discussion.

Using the adaptive biotech Immunoseq platform to cross-reference our TCR sequences in other published studies that used the Immunoseq platform, we identified *DLGAP1* MANA-specific TCR sequence was found infiltrating pre- or post-immunotherapy treatment tumor specimens in 4 of 40 melanoma patients from Dr. Ribas' melanoma immunotherapy cohorts at UCLA[37,38]. We also located our *TTN* MANA-specific TCR sequence infiltrating tumors in

melanoma patients (7 of 40 patients) from Ribas' cohorts[37,38]. In contrast to the *DLGAP1*-MANA TCR, we were able to observe the presence of *TTN* MANA-specific *TCR* sequences in matching pre- and post-immunotherapy patient tumor specimens, in some cases increased frequency was seen in matching post-therapy tumors (**Figure 2)**.

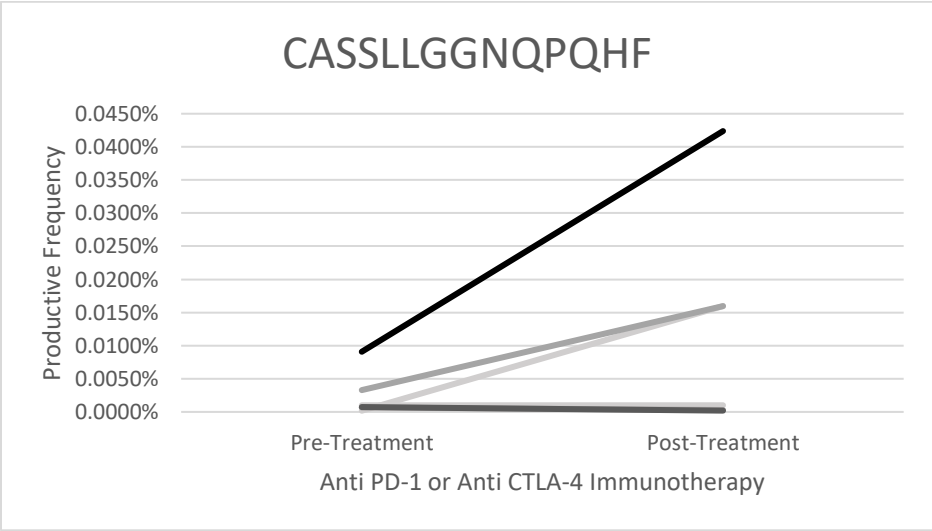

Figure 2: **Frequency of CASSLLGGNQPQHF TCRVβ CDR3 sequences in other patients:** TCR sequences from pre- and post-immunotherapy tumor infiltrate in melanoma patients from cohorts operated by Antoni Ribas M.D., Ph.D. at UCLA. Sequence was found in samples from 11 of 40 patients, and in 5 of these 11 patients we found this sequence in matched pre- and post- immunotherapy as seen in graph.

E. Discussion

While many studies have investigated patient antitumor responses to checkpoint blockade immunotherapy in the context of autologous tumor antigen expression or $CD8^+$ T cell repertoire, there are few which simultaneously evaluate both. Using our specialized FEST assay, we characterized functionally responsive $CD8^+$ T cells concurrently with their native TCR antigen-specificity.

Failure to identify antigen-specific expansion in T cell cultures from two of the melanoma patients could be due to the lower number of peptides tested in their cultures due to lower T cell yield from PBMC samples (42 and 15 peptides tested in patients 1 and 2 respectively, compared to 65 peptides tested in patient 3). Since FEST analysis evaluates plates individually, and peptides conditions are not tested in duplicate (due to limited patient samples), statistical power to detect significant antigen-specific expanded T cell clone populations is generated, in part, by the number of different peptide conditions tested per plate.

T cell cultures from patient 3 shows the presence of TAA- and MANA-specific $CD8^+$ T cells peripherally circulating 3 years after their last cycle of immunotherapy treatment. After failing to locate our identified antigen-specific TCRs within autologous tumor infiltrate, we speculate that adequate TCR sequencing read depth was not reached in isolated TIL DNA[34]. Repeat TCR sequencing of more appropriately stored and processed tumor specimens may provide sufficient read depth, allowing for higher accuracy in TCR sequencing. We did however,

locate a few of our identified antigen-specific TCR sequences in the tumor infiltrate of other melanoma patients from a separate cohort, which will be discussed shortly.

Many of the MANA peptides which produced antigen-specific TCR expansions were derived from proteins with various clinical or pathological relevance to melanoma. With this respect, TCR clonotypes carrying specificities to *DLGAP1, TTN,* and *HUWE1* MANAs will be discussed further.

DLGAP1 encodes for guanylate kinase-associated protein, discs large-associated protein1, which primarily functions as a scaffold protein in neuronal synapses, where it contributes to homeostasis of synaptic excitation through regulation of post-synaptic Ca^{2+} levels[35]. A study analyzing single nucleotide polymorphisms (SNPs) of germline susceptibility genes in 891 melanomas, *DLGAP1* was found to be one of the two germline genes where SNPs significantly predict decreased recurrence-free survival and overall-survival in metastatic melanoma patients[36]. As of our *DGLAP1*-MANA may potentially be displayed in two different patient HLA-allele restrictions (711 E>G, HLA-C*4:01 or HLA-C*8:02), this may show possible preference for presentation of this antigen. We also detected the resulting *DLGAP1* MANA-specific TCRVβ CDR3 amino acid sequence (CASSYLTGELFF) in tumor specimens of other melanoma patients from a separate melanoma cohort. Although observed at low frequencies in these patients (<.001% of all productive TCR templates), the presence of MANA-specific TCR sequences signifies potential shared MANA expression, which are generally considered to patient-specific[25].

TTN, the largest gene in the human genome, encodes for the protein titin (connectin), which is involved with contraction of striated muscle[39]. Stimulation with *TTN*-MANA peptide

(4247E>K, HLA-C*8:02 restricted) produced the largest and most significant TCR clone expansion of all MANA-/TAA-specific TCR clones. High somatic mutation frequency (32%) at the same location, alongside intense clonal expansion of its antigen-specific TCR clonotype (CASSLLGGNQPQHF), may signify potential T cell preference in recognizing the *TTN* protein or this MANA. A meta-analysis performed on tumors from 241 melanoma patients found the *TTN* gene to be the most significantly associated mutated gene (64.6%, p=.009) in tumors also containing BRAF mutations[40]. Since up to 60% of melanoma patients have tumors with BRAF mutations, these findings suggest potential overlap of acquired somatic mutations in the *TTN* gene between patients[41].

HUWE1, an E3 ligase, primarily functioning in polyubiquitinating anti-apoptotic protein MCL1 for subsequent degradation, has recently identified as a tumor suppressor gene in colon cancer[42,43]. Mutations in *HUWE1* may be highly penetrant in tumor distribution, as it may provide critical survival advantages for the tumor. We observed two separate TCR clonotypes expand in the same *HUWE1* MANA-peptide culture, producing the highest cumulative frequency of all expanded antigen-specific TCRs, suggesting the importance of developing two separate CD8+ T cell clones against this *HUWE1* mutation. Although we could not locate these TCR sequences in Ribas' cohort, it recognizes a nonsynonymous mutation which has previously been identified to occur in melanoma (115S>F)[44]. Since this MANA peptide is presented in context of HLA-A*2:01 restriction, one of the most commonly expressed HLA-alleles in humans, there is increased possibility that other patients display the same MANA-peptide and can be recognized by similar/same sequences as our identified TCR clonotype.

For TAA-specific TCR clonotypes, two separate amino acid TCR sequence clones significantly expand to the same MART-1 peptide (HLA-A*2:01) while single TCR clonotypes expanded in two different PMEL (gp100) peptide cultures. Our identification of TAA-specific CD8+ T cell clones provides insight on antitumor TCR specificities in patients who receive combined checkpoint blockade immunotherapy and TAA T cell targeting vaccines.

In addition to identifying TAA- and MANA-specific amino acid TCR clonotypes, we highlight the observation of multiple clonal T cell populations of differing underlying nucleotide sequences contributing to the same encoded amino acid sequence, with one nt TCR clone dominating in contribution to antigen-specific expansions of the same amino acid TCR clonotype. We speculate that the presence of multiple CD8+ T cell clones carrying the same TCR specificity may display a certain level of TCR target recognition preference in types of antigens displayed on MHC molecules. Amino acid TCR sequences which recognized clinically relevant peptides, *HUWE1, DLGAP1, TTN*, *MART-1, and PMEL* all received contributions from least 3 populations of different nt TCR sequence clones.

In summary, we demonstrate the presence of CD8+ T cells targeting both types of tumor antigens, TAAs and MANAs, in circulating peripheral blood of patients 3 years after their last treatment with checkpoint blockade immunotherapy. Recognizing that CD8+ T cell memory develops after prolonged antigen stimulation and resultant CD8+ T cell activation, our functionally expanded TCR clonotypes to tumor antigen stimulation may represent long-lived antitumor CD8+ T cell memory. The identification of these tumor antigen-specific CD8+ TCRs in patients who had complete responses to immunotherapy, suggests that effective antitumor immune responses have endogenous CD8+ TCR repertoire specificities for both TAAs and

MANAs. With multiple TCR amino acid clonotypes and multiple contributing nt TCR clones functionally carrying specificities for tumor antigens, we speculate that these may be characteristics of the magnitude of T cell expansion. The breadth of tumor antigen recognizing TCRs and the clinical relevance of the proteins they're derived from, may imply strength and persistence in T cell targeting of these antigens. We also demonstrate TCR recognition of a recurrent melanoma mutation (*HUWE1,* 115S>F) and presence of MANA-specific TCR sequences infiltrating tumors from different patients.

Cancer immunotherapy continues to be one of the most rapidly expanding cancer research fields, with focus shifting towards targeting tumor antigens to synergistically activate immune responses. However, not all tumor antigens are created equal, to be a bona fide tumor regression antigen, it must fulfill specified requirements: 1) Selectively expressed in tumor 2) Shared between patients 3) Persist despite immune pressure[45]. Although more validation studies are required, our initial observations of patient antigen-specific TCR clonotypes, show targeting tumor antigen which potentially fulfill these three ideal conditions.

F. Future Directions

After the identification of antigen-specific TCRs which recognize TAAs and MANAs, we will use software developed by our lab (IMMUNOMAP) to assess amino acid peptide motifs they recognize.

We also hope to repeat the FEST assay with available PBMCs from equivalent timepoints in patients which we failed to detect functional T cell expansions. To validate MANA-specific TCR clones, we will stimulate patient 3 T cells with wild-type peptide forms. We also plan on acquiring PBMC samples from the same patients at different timepoints, so that we may map the frequency of our antigen-specific TCR clones throughout the patient's course of immunotherapy.

We will also assess immunological markers expressed in the tumor microenvironment, using immunohistochemistry of tumor specimens. These markers will help determine what immune checkpoints were active in tumor environment. Staining will determine differentiation of T cell subsets TILs, including tumor infiltrating CTLs.

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H. Supplementary Tables

Supplementary Table 1: Selected validated peptides of known melanoma TAAs.

Peptide ID	TAA protein	Peptide Sequence	HLA Restriction
A201_1	Melanoma antigen recognized by T-cells 1 (MART-1)	AAGIGILTV	HLA-A*2:01
A201_2	Melanocyte protein Pmel 17 precursor	YLEPGPVTA	HLA-A*2:01
A201_3	Melanoma antigen recognized by T-cells 1 (MART-1)	ALMDKSLHV	HLA-A*2:01
A201_4	Tyrosinase precursor	MLLAVLYCL	HLA-A*2:01
A201_5	Tyrosinase	YMDGTMSQV	HLA-A*2:01
A201_6	Cancer/testis antigen 1	SLLMWITQC	HLA-A*2:01
A201_7	MAGEA1	KVLEYVIKV	HLA-A*2:01
A201_8	Melanocyte protein PMEL (gp100)	ITDQVPFSV	HLA-A*2:01
A201_9	Melanocyte protein PMEL (gp100)	KTWGQYWQV	HLA-A*2:01
A201_10	Melanocyte protein PMEL (gp100)	MLGTHTMEV	HLA-A*2:01

Supplementary Table 2: Patient 1 putative MANA-peptides cultured and original somatic peptide sequence.

Peptide ID	HLA Restriction	MANA-Peptide Sequence	Gene	Full Length Somatic Peptide
P1_01	HLA-A*02:01	LLSPHLLLQL	FASN	SRIPGLLSPH[P/L]LLQLSYTATD
P1_02	HLA-A*02:01	YQWDDPDHRL	FASN	GKVYQWDDPD[P/H]RLFDHPESPT
P1_03	HLA-A*02:01	LLSPHLLL	FASN	SRIPGLLSPH[P/L]LLQLSYTATD
P1_04	HLA-A*02:01	LLLQLSYTA	FASN	SRIPGLLSPH[P/L]LLQLSYTATD
P1_05	HLA-A*02:01	YILELAKSLKV	CDC37	QVAHQTIVMQ[F/Y]ILELAKSLKV
P1_06	HLA-A*02:01 HLA-C*03:03	IVMQYILEL	CDC37	QVAHQTIVMQ[F/Y]ILELAKSLKV
P1_07	HLA-A*02:01	VMQYILEL	CDC37	QVAHQTIVMQ[F/Y]ILELAKSLKV
P1_08	HLA-A*02:01	YILELAKSL	CDC37	QVAHQTIVMQ[F/Y]ILELAKSLKV
P1_09	HLA-A*02:01	ILLREVTIV	TBC1D9B	KEEDACHLII[P/L]LREVTIVEKA
P1_10	HLA-A*02:01	KLAMLTRV	BZW1	SERNKLAMLT[G/R]VLLANGTLNA
P1_11	HLA-B*15:01	TQIQSVKPY	STAT3	ISGKTQIQSV[E/K]PYTKQQLNNM
P1_12	HLA-B*15:01	LQSSVLTM	TNRC18	ESKTKNAALQ[P/S]SVLTMCNGGA
P1_13	HLA-B*15:01	YMWGKYDIVF	RNPEPL1	ERLYGPYMWG[R/K]YDIVFLPPSF
P1_14	HLA-B*15:01	IQIKFVRGGTF	POSTN	KYIQIKFVRG[S/G]TFKEIPVTVY
P1_15	HLA-B*15:01	MTMRSLLRTSF	MCFD2	MTMRSLLRT[P/S]FLCGLLWAFC
P1_16	HLA-B*15:01	TMRSLLRTSF	MCFD2	MTMRSLLRT[P/S]FLCGLLWAFC

P1_17	HLA-B*15:01	LQRSGMYTVAM	PSMD1	SLCRDKDPIL[R/Q]RSGMYTVAMA
P1_18	HLA-B*15:01	LMSMSIAPL	POLR2B	QELMSMSIAP[R/L]MMSV*
P1_19	HLA-B*15:01	LMSMSIAPLMM	POLR2B	QELMSMSIAP[R/L]MMSV*
P1_20	HLA-B*15:01	LMSMSIAPLM	POLR2B	QELMSMSIAP[R/L]MMSV*
P1_21	HLA-B*44:02	AEASTGKTPNL	TNC	PVLSAEASTG[E/K]TPNLGEVVVA
P1_22	HLA-B*44:02	REQMKSESW	NARS	WHREQMKSES[R/W]EKKEAEDSLR
P1_23	HLA-B*44:02	EQMKSESW	NARS	WHREQMKSES[R/W]EKKEAEDSLR
P1_24	HLA-B*44:02	SEVAKFCF	GGA2	EKFHSEVAKF[R/C]FLNELIKVLS
P1_25	HLA-B*44:02	EELRPTLF	GGA2	ALQVVYERCE[K/E]LRPTLFRLAS
P1_26	HLA-B*44:02	VELAASSPELY	MEGF8	GFRFHVELAA[P/S]SPELYSLHCP
P1_27	HLA-B*44:02	TEMSLHAFY	ATG9A	YASTEMSLHA[L/F]YMHQLHKQQA
P1_28	HLA-B*44:02	TEMSLHAF	ATG9A	YASTEMSLHA[L/F]YMHQLHKQQA
P1_29	HLA-B*44:02	TEMSLHAFYM	ATG9A	YASTEMSLHA[L/F]YMHQLHKQQA
P1_30	HLA-B*44:02	TEMSLHAFYMH	ATG9A	YASTEMSLHA[L/F]YMHQLHKQQA
P1_31	HLA-C*03:03	SATSGSGAPL	NRP2	SSATSGSGAP[S/L]TDKEKSWLYT
P1_32	HLA-C*03:03	YQATGGRGVVL	NRP2	QYQATGGRGV[A/V]LQVVREASQE
P1_33	HLA-C*03:03	YVQGNDLEL	ITGA3	QRRMVGKCYV[R/Q]GNDLELDSSD
P1_34	HLA-C*03:03	VAVVVNNPL	HMCN1	NMVAVVVNNP[V/L]RLECEARGIP
P1_35	HLA-C*03:03	MVAVVVNNPL	HMCN1	NMVAVVVNNP[V/L]RLECEARGIP

P1_36	HLA-C*03:03	FAFPDKFVF	GIPC1	ERLGDFAFPD[E/K]FVFDVWGAIG
P1_37	HLA-C*03:03	AALQSSVLTM	TNRC18	LAGHPYGLGP[P/S]SLHQGMAPAF
P1_38	HLA-C*03:03	YGLGPSSL	TNRC18	ESKTKNAALQ[P/S]SVLTMCNGGA
P1_39	HLA-C*03:03	AALQSSVL	TNRC18	LAGHPYGLGP[P/S]SLHQGMAPAF
P1_40	HLA-C*05:01	LTDKEKSWL	NRP2	SSATSGSGAP[S/L]TDKEKSWLYT
P1_41	HLA-C*05:01	ITDMGTQEDGV	ITPKB	ITDMGTQEDG[A/V]LEETQGSPRG
P1_42	HLA-C*05:01	LLDMIPPRSIL	GATAD2A	LLDMIPPRSI[P/L]QSATWK*

Supplementary Table 3: Patient 2 putative MANA-peptides cultured and original somatic sequence.

Peptide ID	HLA Restriction	MANA-Peptide Sequence	Gene	Full Length Somatic Peptide
P2_01	HLA-A*01:01	CSDMSCPNDCY	TNC	CSDMSCPNDC[H/Y]QHGRCVNGMC
P2_02	HLA-A*01:01	MSCPNDCY	TNC	CSDMSCPNDC[H/Y]QHGRCVNGMC
P2_03	HLA-A*01:01	GTSKLSSY	DNM2	IQELINTVRQ[C/G]TSKLSSYPRL
P2_04	HLA-A*01:01	ILDRDNIFVY	USP11	EPLSSILDRD[D/N]IFVYEVSGRI
P2_05	HLA-A*01:01	ATASYDGY	EDC4	PDGTVLATAS[H/Y]DGYVKFWQIY
P2_11	HLA-A*02:01	ILDRDNIFV	USP11	EPLSSILDRD[D/N]IFVYEVSGRI
P2_12	HLA-A*02:01	LLYRLQLEML	SRPX	ARNLLYRLQL[G/E]MLQQAQCGLD
P2_13	HLA-A*02:01	VLATASYDGYV	EDC4	PDGTVLATAS[H/Y]DGYVKFWQIY
P2_14	HLA-A*02:01	FQASLVRFFL	OGFR	GELGLEHFQA[P/S]LVRFFLEETL
P2_21	HLA-B*07:02	SPVYSSSNTV	SCAP	SPASPVYSSS[D/N]TVACHLTHTV
P2_22	HLA-B*07:02	RPQSPGASL	SPEN	LRRPQSPGAS[P/L]SQAERLPSDS
P2_23	HLA-B*07:02	TPLFMTPPLTL	BIRC6	TPLFMTPPLT[P/L]PNEAVSVVIN
P2_24	HLA-B*07:02	LPLFSNAVL	HIP1	FWSVVNRLPL[S/F]SNAVLCWKFC
P2_25	HLA-B*07:02	LPLFSNAV	HIP1	FWSVVNRLPL[S/F]SNAVLCWKFC
P2_26	HLA-B*07:02	IPESLMAL	ZER1	MASD[T/I]PESLMALCTD

Supplementary Table 4: Patient 3 putative MANA-peptides cultured and original somatic sequence.

Peptide ID	HLA Restriction	Somatic AA Sequence	Gene	Full Length Somatic Peptide
P3_01	HLA-A*02:01	HLYSFIEHL	HUWE1	EYSFSRHLYS[S/F]IEHLTTLLAS
P3_02	HLA-A*02:01	KLIGSHSI	UBR4	QQVVKLIGSH[T/S]ISKVTVKIGD
P3_03	HLA-A*02:01	KLIGSHSISKV	UBR4	QQVVKLIGSH[T/S]ISKVTVKIGD
P3_04	HLA-A*02:01	KLLNGPIDV	NFE2L2	SPATLSHSLS[E/K]LLNGPIDVSD
P3_05	HLA-A*02:01	IVLEGLPPL	PELP1	SDSDDSVVIV[P/L]EGLPPLPPPP
P3_06	HLA-A*02:01	VLGDGVPSKV	USP32	IREVLGDGVP[P/S]KVAEVIYCSF
P3_07	HLA-A*02:01	FLFSGTVLENL	ABCC10	QEPFLFSGTV[R/L]ENLDPQGLHK
P3_08	HLA-A*02:01	FLFSGTVL	ABCC10	QEPFLFSGTV[R/L]ENLDPQGLHK
P3_09	HLA-A*02:01	YLSRFSTNV	HEATR3	EIVLKYLSRF[P/S]TNVDLAISVA
P3_10	HLA-A*02:01	YLSRFSTNVDL	HEATR3	EIVLKYLSRF[P/S]TNVDLAISVA
P3_11	HLA-A*33:05	NVKGKRVVMR	PGK1	LSNKLTLDKL[D/N]VKGKRVVMRV
P3_12	HLA-A*33:05	DVVLRKINR	AKAP13	KVDRTVDVVL[L/R]KINRENWCTI
P3_13	HLA-A*33:05	SNRRLKHFLR	LRCH4	NLSNRRLKHF[P/L]RGAARSYDLS
P3_14	HLA-A*33:05	HFLRGAAR	LRCH4	NLSNRRLKHF[P/L]RGAARSYDLS
P3_15	HLA-A*33:05	LLKRCQFYR	SEMA5A	VGLREHVVKI[P/L]LKRCQFYRTR
P3_16	HLA-A*33:05	HVVKILLKR	SEMA5A	VGLREHVVKI[P/L]LKRCQFYRTR

P3_17	HLA-A*33:05	ILLKRCQFYR	SEMA5A	VGLREHVVKI[P/L]LKRCQFYRTR
P3_18	HLA-A*33:05	NAVWILWGR	AMBRA1	MKVVPEKNAV[R/W]ILWGRERGAR
P3_19	HLA-A*33:05	NAVWILWGRER	AMBRA1	MKVVPEKNAV[R/W]ILWGRERGAR
P3_20	HLA-A*33:05	IYYQIKQPR	DOCK1	SEYKSVIYYQ[V/I]KQPRWFETVK
P3_21	HLA-B*14:02 HLA-C*04:01	YSFSRHLYSF	HUWE1	EYSFSRHLYS[S/F]IEHLTTLLAS
P3_22	HLA-B*14:02	NRRLKHFL	LRCH4	NLSNRRLKHF[P/L]RGAARSYDLS
P3_23	HLA-B*14:02	EMYNRPVEM	OTUD5	MAEMYNRPVE[V/M]YQYSTGTSAV
P3_24	HLA-B*14:02 HLA-C*08:03	SASPHPCSSPL	PRDM2	PSSSASPHPC[P/S]SPLSNATAQS
P3_25	HLA-B*14:02	DRHIPLAM	RASAL2	TVPDRHIPLA[L/M]PRQNSTGQAQ
P3_26	HLA-B*14:02 HLA-B*51:01	MPFFTARL	UBOX5	ALASTLGSMP[S/F]FTARLTRGQL
P3_27	HLA-B*14:02	LRFLHTRL	KCNS3	SVDQSTLLRF[P/L]HTRLGKLLTC
P3_28	HLA-B*14:02	LRFLHTRLGKL	KCNS3	SVDQSTLLRF[P/L]HTRLGKLLTC
P3_29	HLA-B*14:02	HKMYREQINL	FAM20A	NRRHKMYREQ[M/I]NLTSLDPPLQ
P3_30	HLA-B*14:02	SRHLYSFIEHL	HUWE1	EYSFSRHLYS[S/F]IEHLTTLLAS
P3_31	HLA-B*51:01	YSFSRHLYSFI	HUWE1	EYSFSRHLYS[S/F]IEHLTTLLAS
P3_32	HLA-B*51:01	CPFGAESNWSL	TNKS1BP1	QGQGSQLALD[R/C]PFGAESNWSL
P3_33	HLA-B*51:01	VPSKVAEVI	USP32	IREVLGDGVP[P/S]KVAEVIYCSF

P3_34	HLA-B*51:01	VPSKVAEV	USP32	IREVLGDGVP[P/S]KVAEVIYCSF
P3_35	HLA-B*51:01	MPRLVSYHGCV	KLHL24	IITGVAAMPR[P/L]VSYHGCVTIH
P3_36	HLA-B*51:01	LPLTMTSV	ZHX3	QATSSPLPLT[V/M]TSVPKQPGVA
P3_37	HLA-B*51:01	SPLPLTMTSV	ZHX3	QATSSPLPLT[V/M]TSVPKQPGVA
P3_38	HLA-B*51:01	FSLEPKFAKTI	DHX33	TPMGRKMAAF[P/S]LEPKFAKTIL
P3_39	HLA-B*51:01	MPGFFVPTV	FOSB	ECAGLGEMPG[S/F]FVPTVTAITT
P3_40	HLA-C*04:01	FYEWFLRTF	ATR	FLLPRHPPIF[H/Y]EWFLRTFPDP
P3_41	HLA-C*04:01	YREQINLTSL	FAM20A	NRRHKMYREQ[M/I]NLTSLDPPLQ
P3_42	HLA-C*04:01	SFDSRRFDF	FCGBP	SGDPHYVSFD[G/S]RRFDFMGTCT
P3_43	HLA-C*04:01	SFDSRRFDFM	FCGBP	SGDPHYVSFD[G/S]RRFDFMGTCT
P3_44	HLA-C*04:01 HLA-C*08:02	FHDNLGNSL	DLGAP1	QADLDFHDNL[E/G]NSLESIEDNS
P3_45	HLA-C*04:01	YRLDSSSPHTF	SDK1	ILGYQIAYRL[A/D]SSSPHTFTTV
P3_46	HLA-C*04:01 HLA-C*08:02	RLDSSSPHTF	SDK1	ILGYQIAYRL[A/D]SSSPHTFTTV
P3_47	HLA-C*04:01	KRDASGSMWL	CPAMD8	RQDGSYSAFG[E/K]RDASGSMWLT
P3_48	HLA-C*04:01	FYTVVTTLL	OR4E2	VVSVFYTVVT[P/T]LLNPFIYTLR
P3_49	HLA-C*08:02	FIEHLTTL	HUWE1	EYSFSRHLYS[S/F]IEHLTTLLAS
P3_50	HLA-C*08:02	FSTNVDLAI	HEATR3	EIVLKYLSRF[P/S]TNVDLAISVA
P3_51	HLA-C*08:02	SVDQSTLLRFL	KCNS3	SVDQSTLLRF[P/L]HTRLGKLLTC

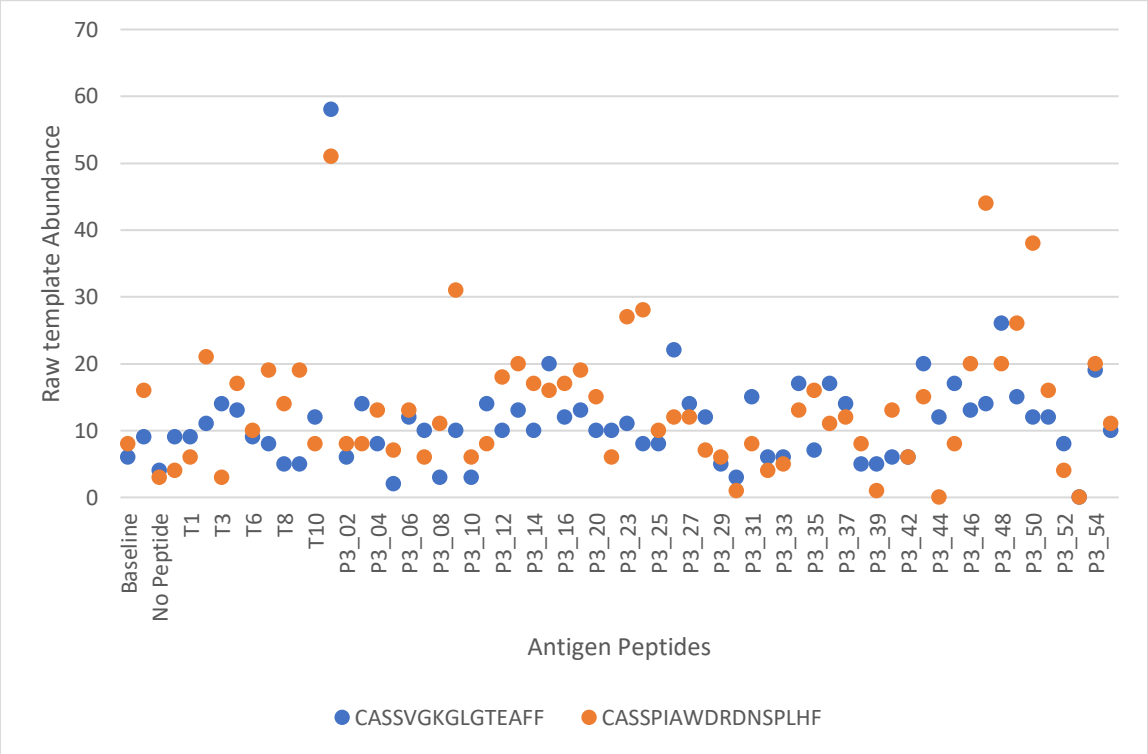
P3_52	HLA-C*08:02	VSDKKQDESL	TTN	KGVASAVVSD[E/K]KQDESLKPSE

Supplementary Table 5: Contributing nucleotide TCR sequences and frequencies to amino acid TCR clonotypes.

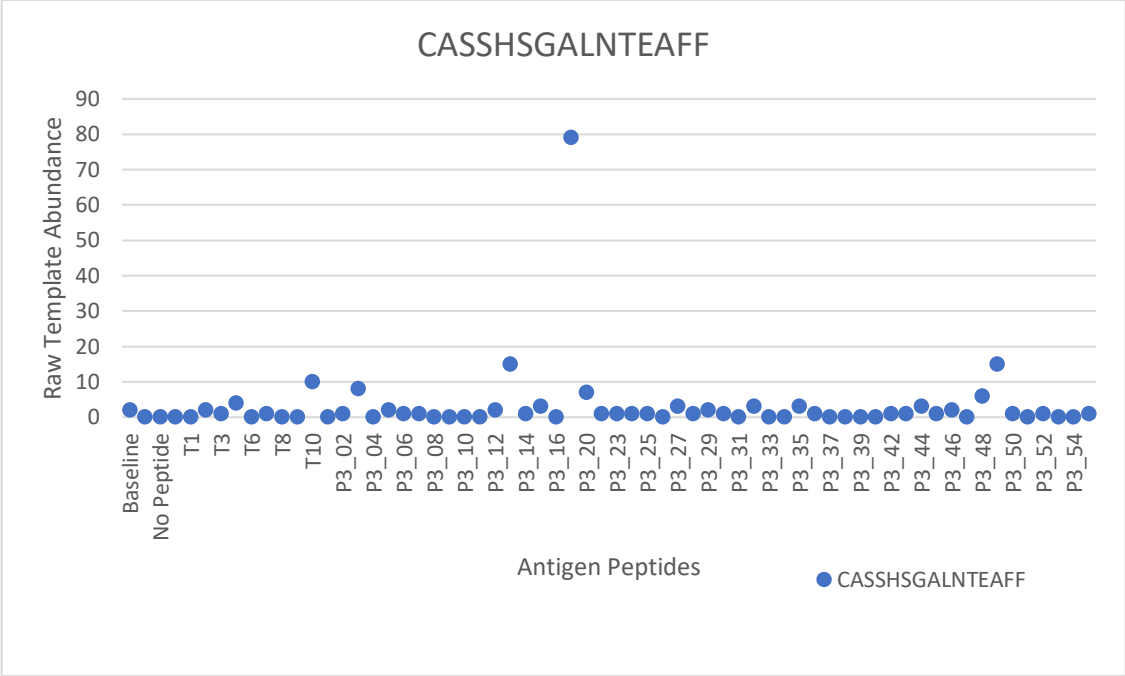
Peptide	Template Abundance	TCR template frequency %	TCRVβ CDR3 Nucleotide Sequence
TTN	78	0.0054363	CTGGAGTCGCCCAGCCCCAACCAGACCTCTCTGTA CTTCTGTGCCAGCAGTTTGCTGGGAGGCAATCAG CCCCAGCATTTTGGTGAT
TTN	1	6.97E-05	CTGGAGTCCGCCAGCACCAACCAGACATCTATGTA CCTCTGTGCCAGCAGTTTGCTGGGAGGCAATCAG CCCCAGCATTTTGGTGAT
TTN	1	6.97E-05	CCTGGAGTCGCCCAGCCCAACCAGACCTCTCTGTA CTTCTGTGCCAGCAGTTTGCTGGGAGGCAATCAG CCCCAGCATTTTGGTGAT
TTN	1	6.97E-05	TTGGAGTCGGCTGCTCCCTCCCAAACATCTGTGTA CTTCTGTGCCAGCAGTTTGCTGGGAGGCAATCAG CCCCAGCATTTTGGTGAT
TTN	1	6.97E-05	CCTGGAGTCGCCAGCCCCAACCAGACCTCTCTGTA CTTCTGTGCCAGCAGTTTGCTGGGAGGCAATCAG CCCCAGCATTTTGGTGAT
CPAMD 8	47	0.0016292	CTGAAGATCCAGCCCTCAGAACCCAGGGACTCAG CTGTGTACTTCTGTGCCAGCAGTTTTCGTATGCCT GAAGCTTTCTTTGGACAA
DGLAP1	33	0.002099	CTCAAGATCCAGCCTGCAGAGCTTGGGGACTCGG CCGTGTATCTCTGTGCCAGCAGCTACCTAACCGGG GAGCTGTTTTTTGGAGAA
DGLAP1	2	0.00012721	CTCAAGATCCAGCCTGCAAAGCTTGAGGACTCGG CCGTGTATCTCTGTGCCAGCAGCTACCTAACCGGG GAGCTGTTTTTTGGAGAA
DGLAP1	1	6.36E-05	TCTCAAGATCCAGCCTGCAGAGCTTGGGACTCGG CCGTGTATCTCTGTGCCAGCAGCTACCTAACCGGG GAGCTGTTTTTTGGAGAA
ZHX3	51	0.0037552	TCAGAACCCAGGGACTCAGCTGTGTACTTCTGTGC CAGCAGTTTAGCGCTATCCGAAACAAATAACTATG GCTACACCTTCGGTTCG
ZHX3	1	7.36E-05	GCTGCTCCCTCCCAGACATCTGTGTACTTCTGTGC CAGCAGTTTAGCGCTATCCGAAACAAATAACTATG GCTACACCTTCGGTTCG
ZHX3	1	7.36E-05	CCCAGCCCCAACCAGACCTCTCTGTACTTCTGTGC CAGCAGTTTAGCGCTATCCGAAACAAATAACTATG GCTACACCTTCGGTTCG

SEMA5A	78	0.0039084	CAGCCTGCAGAACTGGAGGATTCTGGAGTTTATTTCTGTGCCAGCAGCCATTCGGGTGCCCTTAACACTGAAGCTTTCTTTGGACAA
SEMA5A	1	5.01E-05	AATTCCCTGGAGCTTGGTGACTCTGCTGTGTATTTCTGTGCCAGCAGCCATTCGGGTGCCCTTAACACTGAAGCTTTCTTTGGACAA
HUWE1	1	7.27E-05	GAGCTCTCTGGAGCTGGGGACTCAGCTTTGTATTTCTGTGCCAGCAGCGTAGGCAAGGGGCTTGGCACTGAAGCTTTCTTTGGACAA
HUWE1	57	0.0041421	AGCTCTCTGGAGCTGGGGGACTCAGCTTTGTATTTCTGTGCCAGCAGCGTAGGCAAGGGGCTTGGCACTGAAGCTTTCTTTGGACAA
HUWE1	51	0.0037061	CTGGAGCTTGGTGACTCTGCTGTGTATTTCTGTGCCAGCAGCCCTATAGCATGGGACAGGGACAATTCACCCCTCCACTTTGGGAAC
PMEL	17	0.00152	CCTGCAAAGCTTGAGGACTCGGCCGTGTATCTCTGTGCCAGCAGCCCCGGACAGGGGAGCTCTGGAAACACCATATATTTTGGAGAG
PMEL	1	8.94E-05	CCTGCAGAGCTTGGGGACTCGGCCGTGTATCTCTGTGCCAGCAGCCCCGGACAGGGGAGCTCTGGAAACACCATATATTTTGGAGAG
PMEL	1	8.94E-05	CGCACACAGCAGGAGGACTCGGCCGTGTATCTCTGTGCCAGCAGCCCCGGACAGGGGAGCTCTGGAAACACCATATATTTTGGAGAG
MART-1	33	0.0016424	GTGACCAGTGCCCATCCTGAAGACAGCAGCTTCTACATCTGCAGTGCTAGAGAGGGGACAGCGAACACTGAAGCTTTCTTTGGACAA
MART-1	29	0.0014433	ATCCGGTCCACAAAGCTGGAGGACTCAGCCATGTACTTCTGTGCCAGCAGCACCTCTCATCTGGGGTGGGGCTACACCTTCGGTTCG
PMEL	25	0.0014374	ATCAATTCCCTGGAGCTTGGTGACTCTGCTGTGTATTTCTGTGCCAGCAGCAAACAGGGTGAGGGGGAAAAACTGTTTTTTGGCAGT

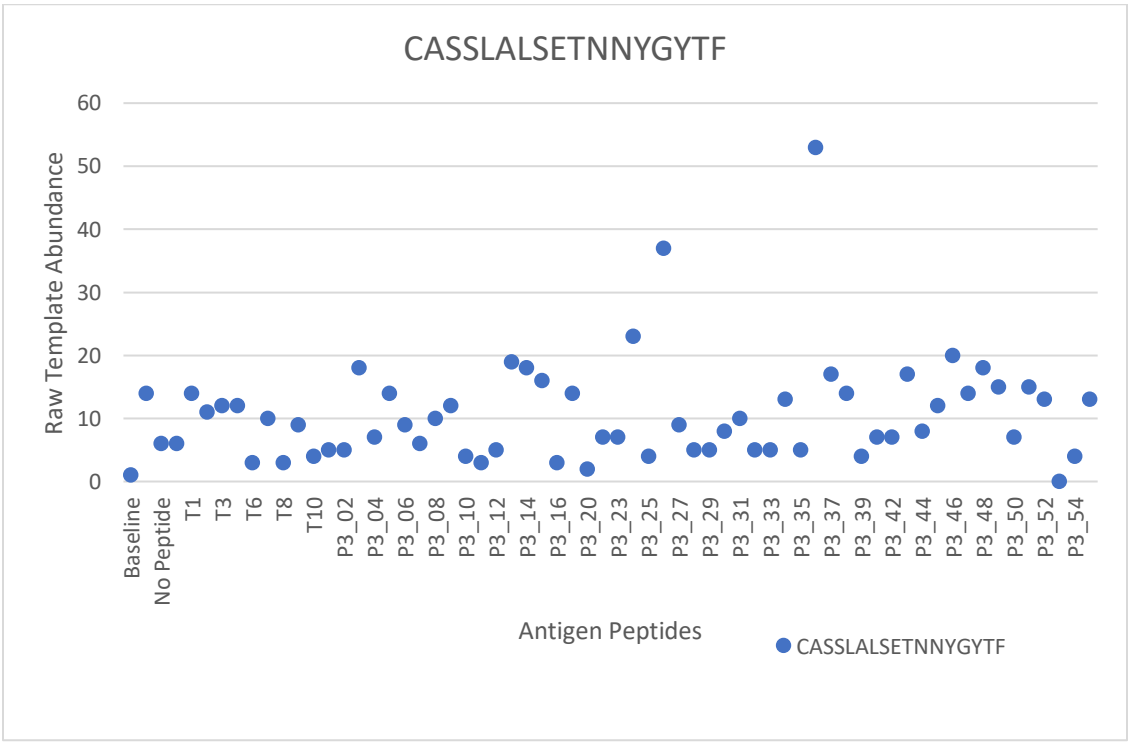
I. Supplementary Figures



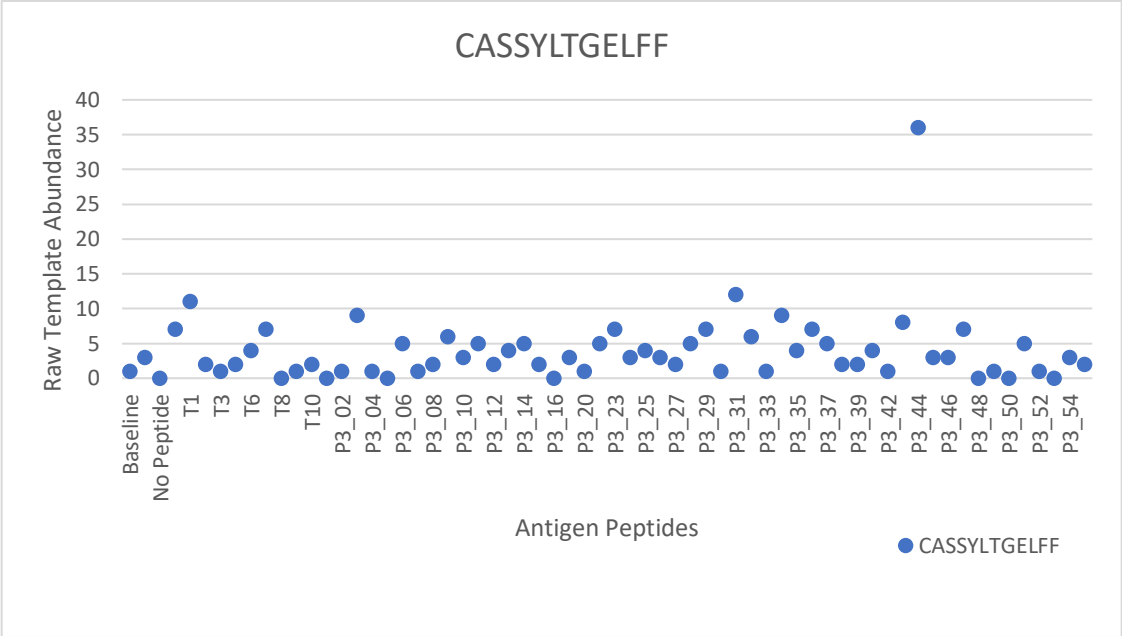
Supplementary Figure 1: Significant antigen-specific expansion of TCR clonotypes to stimulation with *HUWE1* MANA-peptide (SF_01 along the X-axis). Orange circle: TCRVβ CDR3 sequence CASSVGKGLGTEAFF; Blue circle: TCRVβ CDR3 CASSPIAWDRDNSPLHF



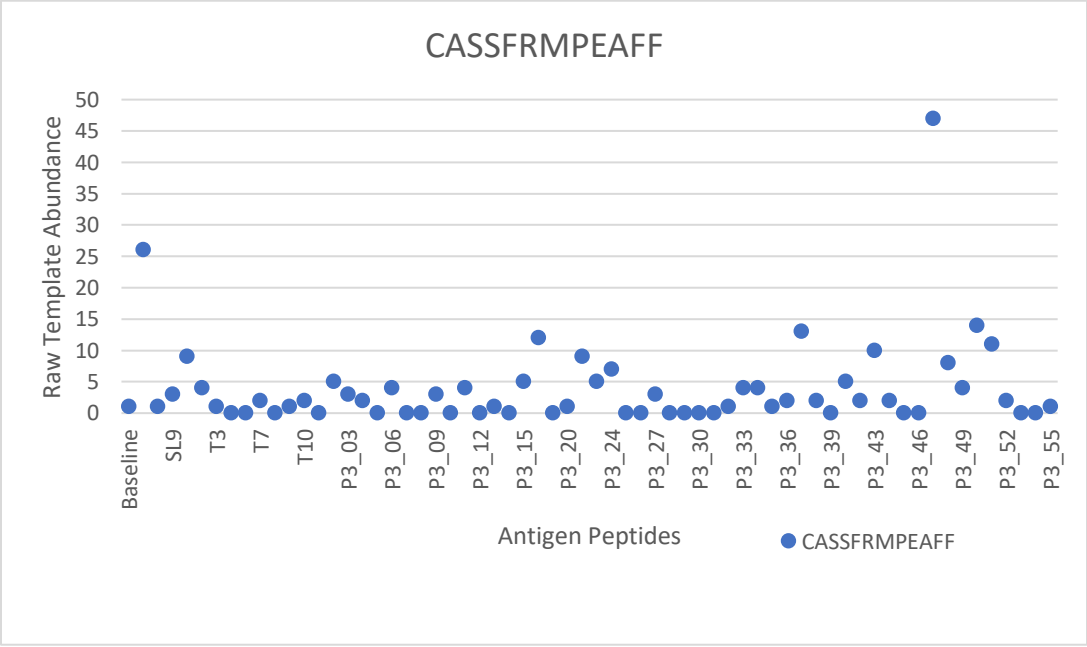
Supplementary Figure 2: Antigen-specific expansion of TCR clonotype (TCRVβ CDR3 sequence: CASSHSGALNTEAFF) to *SEMA5A* MANA-peptide culture (P3_17 peptide).



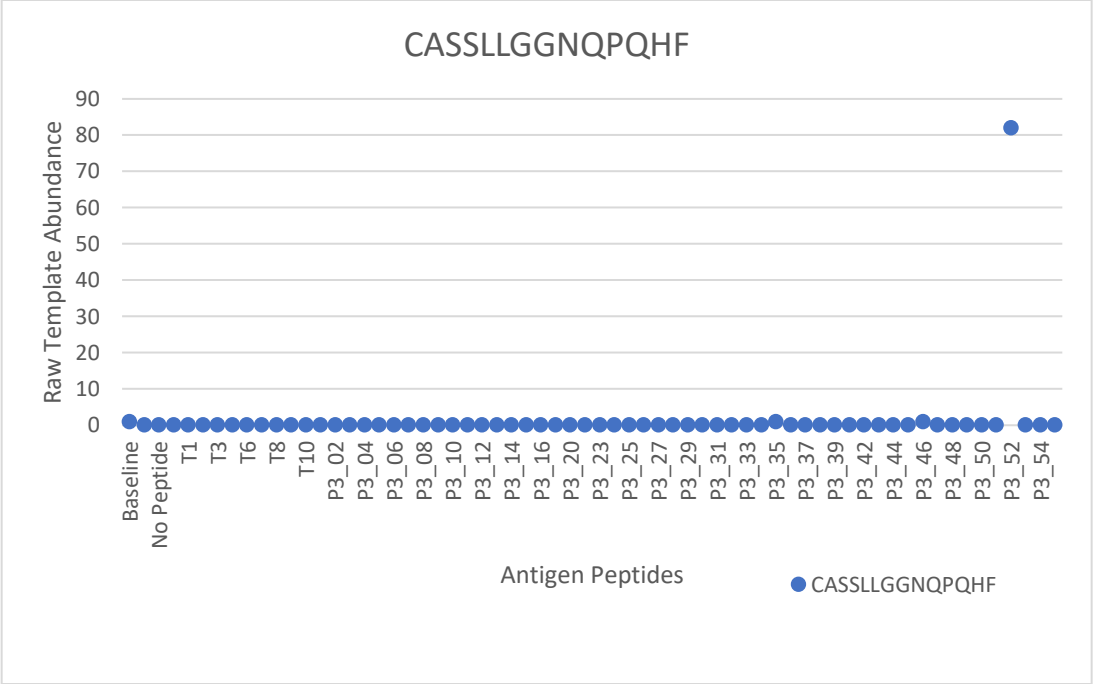
Supplementary Figure 3: Antigen-specific expansion of TCR clonotype (TCRVβ CDR3 sequence: CASSLALSETNNYGYTF) to *ZXH3* MANA-peptide culture (P3_36 peptide).



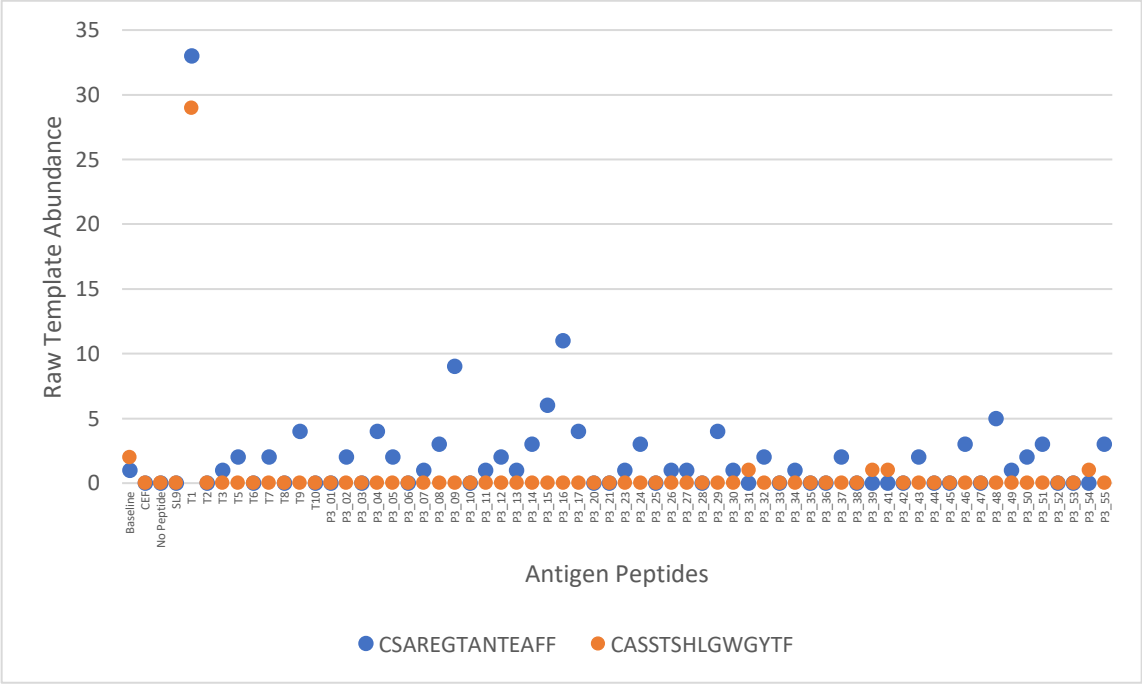
Supplementary Figure 4: Antigen-specific expansion of TCR clonotype (TCRVβ CDR3 sequence: CASSYLTGELFF) to *DLGAP1* MANA-peptide (P3_44).



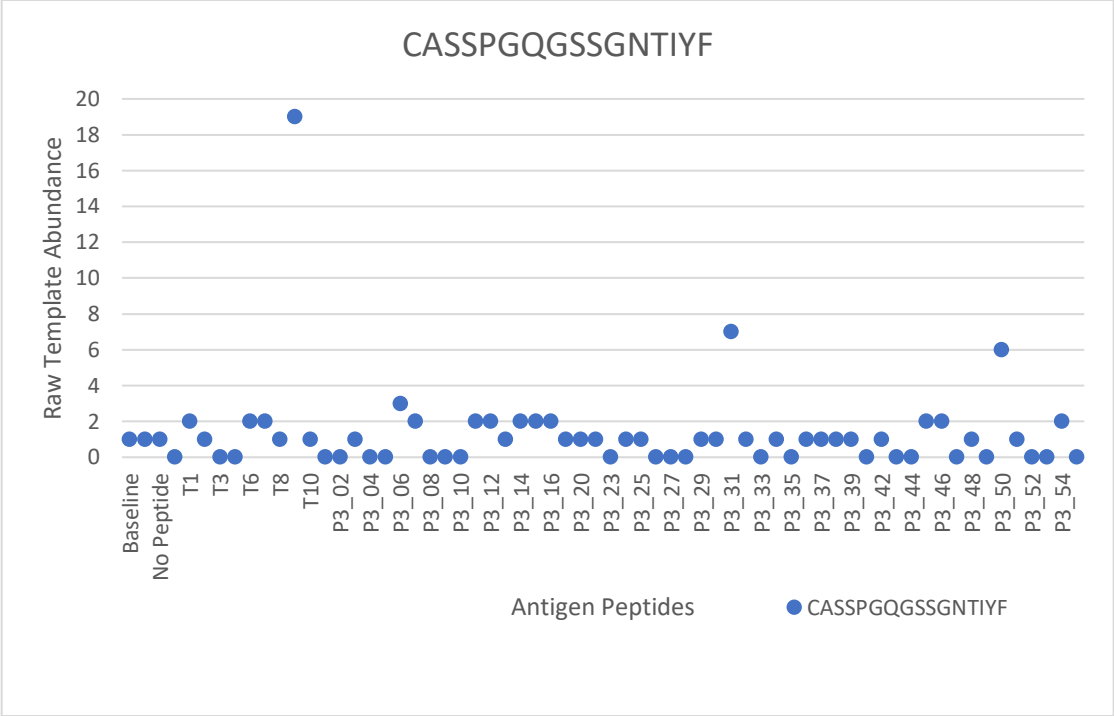
Supplementary Figure 5: Antigen-specific expansion of TCR clonotype (TCRVβ CDR3 sequence: CASSYLTGELFF) to CPAMD28 MANA-peptide (P3_47).



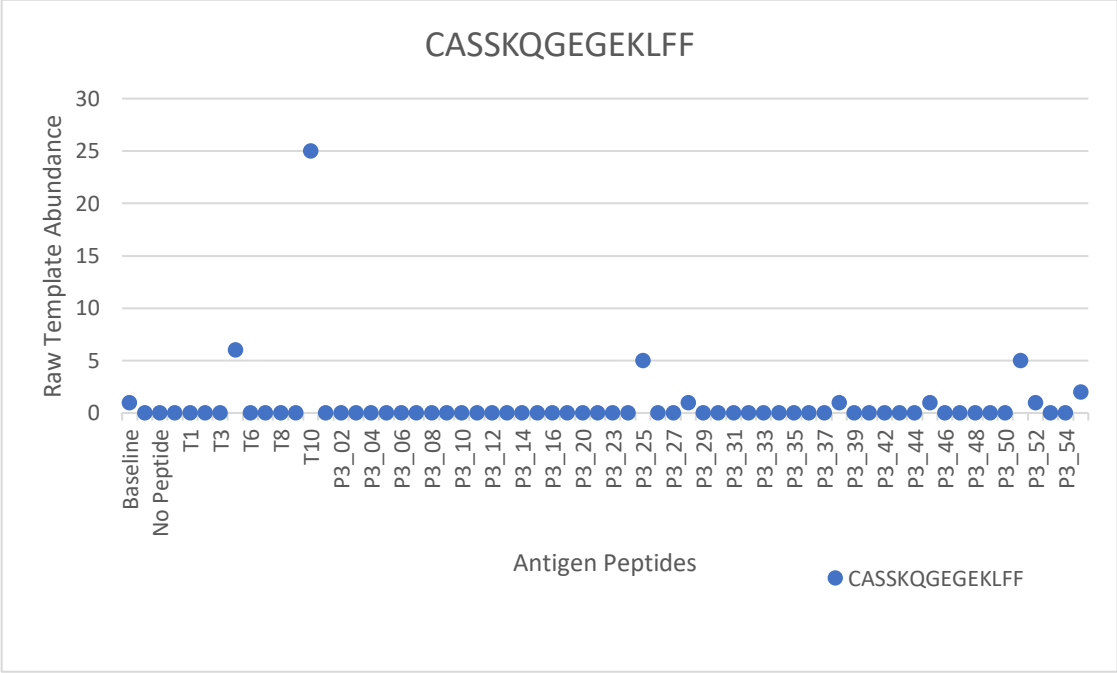
Supplementary Figure 6: Antigen-specific expansion of TCR clonotype (TCRVβ CDR3: CASSLLGGNQPQHF), to *TTN* MANA-peptide (P3_52).



Supplementary Figure 7: Two separate TCR clonotypes (TCRVβ CDR3: Blue circles = CASSVGKGLGTEAFF; Orange circles = CASSTSHLGWGYTF) with significant antigen-specific expansion to MART-1 peptide (T1).

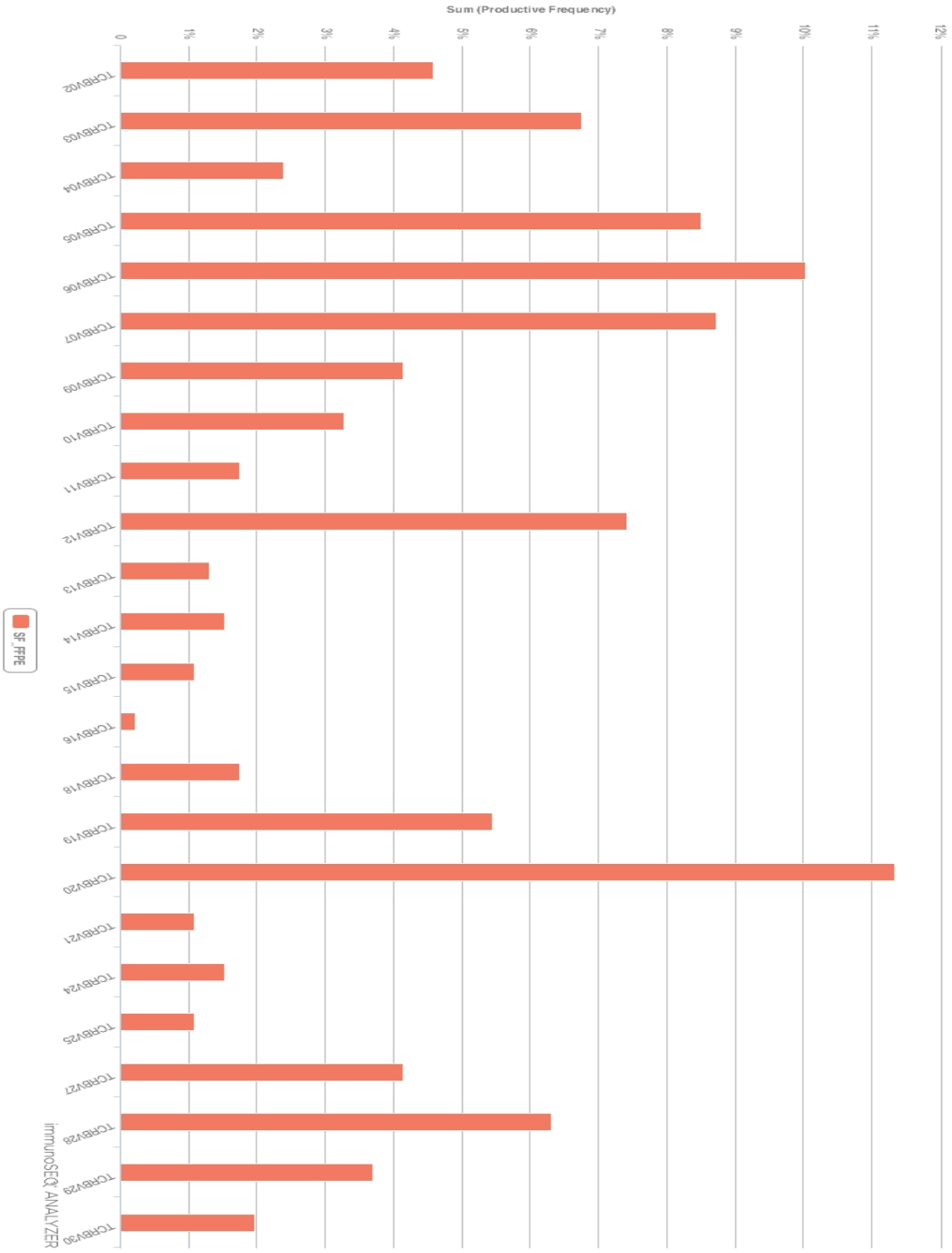


Supplementary Figure 8: Antigen-specific expansion of TCR clonotype (TCRVβ CDR3: CASSPGQSSGNTIYF) to PMEL (gp100) peptide (T9).



Supplementary Figure 9: Antigen-specific expansion of TCR clonotype (TCRVβ CDR3: CASSKQGEGEKLFF) to separate PMEL (gp100) peptide (T10).

Supplementary Figure 10: Patient 3 Intratumor T cell TCRVβ Usage**.** Image adopted from Adaptive Biotechnologies© online Immunseq analysis platform.



Andrew Harold Yang

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EDUCATION

University of Delaware **Newark, Delaware**

Bachelors of Arts in Biology **July 2013**

Johns Hopkins University **Baltimore, Maryland**

Masters of Science **May 2018**

Research/Lab Experience

Johns Hopkins Hospital Thoracic Department of Oncology Research

Baltimore, Maryland

- **Volunteer Lab assistant** **June-August 2009**
  - Principle Investigator: Stephen Yang M.D.
  - Processed patient blood and sputum for further analysis for biomarker research
  - Analyzed data of patients who underwent esophajectomy for benign disease.
- **Undergraduate Research Assistant** **June-August 2012**
  - Principle Investigator: Malcolm Brock M.D.
  - Formed database of patients and corresponding tissue specimens of those who received Metformin while having late stage lung cancer. Immunoglobulins analyzed by Dr. Gabrielson for relevance.
- **Laboratory Assistant:** **March 2014-Present**
  - Principle Investigator: Malcolm Brock M.D.
  - Collected clinical/surgical tumor, blood, sputum, urine research specimens.
  - Further processing of blood, sputum, urine specimens for utilization in later epigenetic testing
  - Performed DNA extraction techniques (MOB) and PCR to analyze presence of epigenetic markers for potential biomarker usage.
  - Optimized blood processing to conserve DNA and RNA content for later analysis.
  - Analyzed data of cohort of 600+ patients to related changes of atmospheric pressure with incident of onset of spontaneous pneumothorax. Also included analysis of predisposing demographic factors that put patients at risk for recurrence

- Assisted in literature research for various grant proposals and research projects

- **Graduate Thesis Research: June 2017 – June 2018**
  - Principle Investigator Drew Pardoll M.D.,Ph.D.
  - Conducted research on thesis project: Long-lived antitumor $CD8^+$ T cells in Melanoma patients who had complete response to checkpoint blockade immunotherapy
  - Processed blood for isolation of peripheral blood mononuclear cells.

University of Delaware, Department of Biology

Newark, Delaware

- **Undergraduate Research Assistant Oct 2012-Jun 2013**
  - Maintained cell culture LN-CAP prostate cancer cell lines to use for flow cytometry. Correlating studies investigated metastasis movement of cancer cells under fluid shear condition.

Christiana Care Hospital Research Department of Cardiology

Newark, Delaware

- **Undergraduate Research Assistant Dec 2010-Jun 2011/Dec 2011-Jun 2012**
  - Formed database of patients receiving CABG surgery. Determined which patients underwent surgery after vasospasms were mistaken as left main coronary artery disease. Article published in Journal of the American College of Cardiology.[1]
  - Database research regarding factors effecting drop-out rates during cardiac rehab. Abstract presented in Washington, D.C.[2]

Publications

1. Mohammed AA, **Yang AH**, Shao K, DiSabatino A., Blackwell R., Banbury M.,Weintraub W. Doorey A.,Patients with Left Main Coronary Artery Vasospasm Inadvertently Undergoing Coronary Artery Bypass Grafting Surgery. *Journal of the American College of Cardiology.* 2013; 61(8): 899-900. doi:10.1016/j.jacc.2012.10.048

2. S. Yang, **A. Yang**, A. Meneshian, M. Brock, M. Sussman, Oesophagectomy for Benign Diseases: Not as Morbid as One Thought.*Interact Cardiovascular Thoracic Surgery 2009;* 91; S92
   a. Presentation by D.E. Wood in Vienna, Austria: Oct. 20 2009.

3. Nellius LM, DiSabatino AJ, **Yang AH**, Furey A, Reed J, Shreve J, Weintraub W. Differences in Gender, Age, and Tobacco Use in the Predication of Successful Completion of Outpatient Cardiac Rehabilitation. *American Heart Association: QCOR in Cardiovascular Disease and Stroke 2011 Scientific Sessions*
    a. Poster presented at *QCOR in Cardiovascular and Stroke 2011 Scientific Sessions* by L.M. Nellius in Washington D.C.: May 13, 2011.

4. Griffin C, Lee B, **Yang AH**, Battafarano V, Rodgers K, Hooker C, Hulbert A, Brock M, Battafarano R, Yang S, Molena D, Trusty D; The Johns Hopkins University TCGA Experience. *International Association for the Study of Lung Cancer:16th World Conference on Lung Cancer*
    a. Poster presented by C. Griffin at 16th World Conference on Lung cancer in Denver, Colorado: September 9, 2015

5. Chen Chen, Anastasia Kottorou, Devlin Danielle, **Andrew Yang**, Kristen Rodgers, Beverly Lee, Candace Griffin, Peter Illei, Peng Huang, Richard Battafarano, Stephen Yang, Fenglei Yu, Tza-Huei Wang, Stephen Baylin, James G. Herman, Alicia Hulbert, Malcolm Brock; DNA Methylation as a Biomarker to Predict Early Recurrence of T1-2N0 Lung Cancer. *American Association for Cancer Research Annual Meeting 2016.*
    a. Poster presented by Chen Chen at AACR Annual Meeting 2016 in New Orleans, Louisiana: April 20, 2016

Abstracts

1. **Yang AH**, Jusue-Torres I, Hulbert A, Battafarano V, Yang SC, Brock M; What are the Risk Factors for Recurrent Spontaneous Pneumothorax?

2. **Yang AH**, Jusue-Torres I, Hulbert A, Hooker C, Battafarano V, Yang SC, Brock M; Does Atmospheric Conditions Effect the Incidence of Spontaneous Pneumothorax.

6. Fotinos-Ioannis D. Dimtrakopoulos, Anastasia Kottorou, **Andrew Yang**, Kristen Rodgers, Stephen Baylin, Alicia Hulbert, Malcolm Brock; Correlation of Exosome Concentrations in the Plasma of Lung Cancer Patients with Disease Stage. *Submitted to the American Society of Clinical Oncology Conference 2016 in Chicago.*

3. A Hulbert, A Stark, I Jusue- Torres, Chen Chen, K Rodgers, B Lee, C Griffin, **A Yang**, P Huang, J Wrangle, S Belinksi, T Wang, S Yang, S Baylin, M Brock, J Herman; Early Detection of Lung Cancer Using DNA Promoter Hypermethylation in Plasma and Sputum.

Biography

Andrew H. Yang was born in 1991 in Houston, Texas. Both his parents are medical professionals, and he an older sister and a twin brother.

Andrew graduated from the University of Delaware in 2013 with a Bachelors of Arts degree in Biology. He was an undergraduate research assistant in Dr. Carlton Cooper's prostate cancer research lab from spring 2011 to spring 2013, helping to culture LN-CaP cell lines for further research. After graduation, he worked in Dr. Brock's lung cancer research lab as a laboratory technician from spring 2013 to 2015 where he assisted on multiple publications and poster projects.

In fall 2016, Andrew matriculated into the MHS program with the Molecular Microbiology and Immunology (MMI) Department at the Johns Hopkins School of Public Health, later transferring into their Sc.M. program. The MMI department allowed him to conduct his thesis with Dr. Drew Pardoll at the Bloomberg-Kimmel Institute for Cancer Immunotherapy in Johns Hopkins School of Medicine.